Correlation of a simple direct measurement of muscle pO_2 to a clinical ischemia index and histology in a rat model of chronic severe hindlimb ischemia

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Purpose: **The lack of suitable experimental models of chronic severe limb ischemia and deficiencies in the available methods that allow for direct intermittent measurement of regional limb perfusion are obstacles to the evaluation of recently developed molecular strategies to reverse severe limb ischemia. Our aim was to develop a model of clinically relevant severe limb ischemia and correlate a simple direct measurement of muscle pO_2 to a clinical ischemia index, muscle mass, and capillary density.**

Methods: **Severe hindlimb ischemia was induced in 44 adult rats with ligation of the left common iliac artery, the femoral artery, and their branches. The effect of ischemia on muscle pO_2 was measured in the left gastrocnemius with room air and with 100% oxygen at 3, 10, 24, and 40 days after ischemia was induced. Clinical ischemia index, muscle mass, cellular proliferation, and capillary density also were assessed.**

Results: **The clinical ischemia index of the left limb was most severe at day 10, with evidence of pressure sores, a pale and dusky limb, and abnormal gait. With the rats breathing room air, muscle pO_2 was significantly lower in the left limbs than in the right limbs at days 3, 10, 24, and 40. After an oxygen challenge (100% O_2), muscle pO_2 was significantly lower at 3, 10, and 40 days. At 3 days, the fraction of muscle mass per total body weight of the left tibialis anterior (TA) was significantly greater than the right TA as a result of edema and inflammation. By days 10, 24, and 40, the left gastrocnemius and TA masses were significantly less than the right as a result of muscle atrophy. Histopathology showed severe necrosis in the left gastrocnemius and TA on day 3. Inflammation was greatest by day 10. Necrotic muscle regenerated but remained atrophic at 40 days. The TA was slower to recover than the gastrocnemius. Capillary densities and capillary-to-muscle fiber ratios were greater in the ischemic limb than in the normal limb at day 24. Cellular proliferation as determined with bromodeoxyuridine labeling reagent staining was maximal in the ischemic limb at day 3.**

Conclusion: **We have developed a rat model of chronic severe hindlimb ischemia with persistent ischemia as shown with a simple direct measurement of muscle pO_2 for up to 40 days. This model of severe hindlimb ischemia may be applicable for future studies of molecular strategies to treat severe limb ischemia in humans. (J Vasc Surg 2002;36:172-9.)**

Peripheral arterial occlusive disease results in significant morbidity and mortality. Surgical revascularization is often effective in reversing symptoms of critical limb ischemia. However, when such procedures fail or cannot be performed, amputation of the affected limb is the only alternative. Molecular strategies of gene therapy to treat and reverse critical limb ischemia in humans are currently being investigated.[1] Gene therapy to treat severe limb ischemia with specific growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, and blood vessel stabilizing molecules, such as angiopoietin-1, offer considerable promise.[2-6]

Animal models have been developed to improve our understanding of this disease and to test these new strategies for gene therapy.[7-11] Although several animal models exist, few have been extensively characterized. Many models of experimental hindlimb ischemia are characterized by rapid normalization of resting blood flow rate and require complex techniques, such as the use of microspheres, radionucleotide perfusion scans, laser scanning, and Doppler measurements, to quantify regional blood flow rates. Although these methods are useful, they are relatively complex to perform consistently and are only indirect measurements of tissue oxygenation. In contrast, tissue oxygen concentration can now be measured simply and directly.[12] We have developed a rat model of hindlimb ischemia that can be assessed with a simple direct measurement of the oxygen tension (pO_2) in the skeletal muscle and have correlated this to a clinical ischemia index, including muscle

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mass and histologic assessment of muscle necrosis, regeneration, inflammation, capillary density, and cellular proliferation.

MATERIALS AND METHODS

Animals. Fifty-three male Sprague-Dawley rats (Simonsen Laboratories, Inc, Gilroy, Calif) weighing 275 to 325 g were studied. Animals were maintained in a room controlled for temperature and light and were provided food and water ad libitum. Animal care complied with the Principles of Laboratory Animal Care (formulated by the National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, DC, National Academy Press, 1996). All protocols were approved in accordance with the Committee on Animal Research at the University of California–San Francisco.

Rat hindlimb ischemia model. Animals were anesthetized for the hindlimb ischemia surgery with 2% isoflurane in 0.5 L/m of oxygen. Animals used for oxygen probe measurements were anesthetized with 35 mg/kg pentobarbital, 0.06 mg/kg buprenorphine hydrochloride, and 0.8 mg/kg atropine. Severe ischemia was induced in the left hindlimb of 44 rats. The contralateral right hindlimb served as a control. Four operated animals died because of anesthetic complications. The left groin and abdominal wall were shaved and cleaned with a betadine solution. Midline abdominal and transverse groin incisions were made. With a surgical operating microscope (Leica Microsystems Inc, Allendale, NJ), the left common iliac artery, the femoral artery, and their associated branches were exposed and ligated with 5-0 silk sutures (US Surgical Corp, Norwalk, Conn). The left common iliac and femoral arteries were transected to ensure that no other collaterals perfused the limb.

Clinical ischemia index. The rats were observed daily for clinical assessment of ischemia. A clinical ischemia index was used to measure the degree of ischemia and function. The animals were scored according to an ischemia grade scale (0 = normal, 1+ = pale, 2+ = pressure sores, and 3+ = necrosis) and gait (limping or no limping)

Oxygen measurements. Muscle pO_2 was measured with the rats breathing room air (RA) or 100% O_2 on days 3, 10, 24, and 40 after ischemia was induced. The measurements of skeletal muscle pO_2 were made with a LICOX Clark type polarographic oxygen probe and temperature measurement system (GMS, Germany) described by Hopf et al.[12] Determination of tissue pO_2 with the polarographic probe was a result of a cathode made slightly negative with respect to the anode with an external voltage source. The dissolved oxygen in the tissue was reduced at the cathode and produced a measurable current. The probe used in our experiment was the Microcatheter pO_2 probe (GMS, Germany), model C1 (outer diameter, 0.47 mm; length, 200 mm; integrating pO_2 sensitive area, 5 mm in length; sensitivity, 2.5×10^{-9} A/mm Hg pO_2; gold cathode polarized to 795 mV). The factory described 90% response time of the probe was 70 seconds at a temperature of 35° C. The sensitivity of the polarographic probe was modulated with temperature (approximately 4.5%/° C). For this reason, tissue temperature was continuously monitored with a thermocouple. The oxygen probe and thermocouple were connected to the LICOX computer, which took into account the temperature when calculating the pO_2. The relationship between pO_2 and temperature was governed with the following equation: pO_2 (Tissue) = pO_2 (cal) (I − IcN_2)/(IcO_2 − IcN_2) exp [.01 TS (Tc-Tt)], where pO_2 *(cal)* is the calculated pO_2 at the time of calibration in an oxygen-containing environment (usually RA); *I* is the polarographic probe current during measurement; IcN_2 is the polarographic probe current with pure nitrogen (an oxygen free environment); IcO_2 is the polarographic probe current at the time of calibration in an oxygen-containing environment (usually RA); *TS* is the temperature coefficient quantitating the current sensitivity to temperature; *Tc* is the temperature at the time of calibration in an oxygen-containing environment (usually RA); and *Tt* is the temperature of the tissue or substance measured.

IcN_2 and TS were supplied with each probe and were determined at the time of manufacture. The probes were calibrated with placement in oxygen permeable polyethylene sheaths in equilibrium with RA. The factory parameters supplied with the probes were entered into the computer. The probes were calibrated with RA conditions with values of the barometric pressure and fractional oxygen content entered into the computer. The probes were allowed to equilibrate for 20 minutes, at which time the calibration button was pressed, simultaneously storing the values of pO_2 (cal), IcO_2, and Tc.

Rats were anesthetized as previously mentioned, and the right and left hindlimbs were shaved and cleaned with a betadine solution. A longitudinal skin incision was made along the gastrocnemius until adequate exposure of the muscle was achieved. Animals were placed in a supine position on a heating pad at 37° C to maintain constant body temperature. A LICOX probe and thermocouple were inserted into the left and right gastrocnemius through 18G spinal needles. The spinal needles then were carefully removed, leaving the probe and thermocouple in the muscles. Muscle pO_2 data were recorded until a plateau was reached. For muscle pO_2 at 100% oxygen, the rats were placed in a sealed transparent plastic chamber that was gradually filled with oxygen until approximately 100% oxygen was achieved as measured with a Capnomac respiration monitor (Datex-Ohmeda, Helsinki, Finland).

Tissue preparation. The rats were killed after pO_2 measurements and the gastrocnemius and tibialis anterior (TA) from both limbs were harvested and weighed for assessment of atrophy. Middle sections of muscle were processed for morphologic analysis to assess the degree of ischemic necrosis and subsequent regeneration, variables not measured in most other studies. Immunohistochemistry also was performed to determine capillary counts.

Approximately 24 hours before death, rats received two intraperitoneal injections (1 mL/100 g body weight), sep-

arated 12 hours apart, of a 10 mmol/L 5-bromo-2′-deoxyuridine labeling reagent (BrdU; Roche Diagnostics Corp, Indianapolis, Ind) in water. A small segment of small intestine served as a positive control. A 5-mm section of each muscle was mounted onto a cork base and secured with a gum tragacanth paste (Sigma, St Louis, Mo). The tissue was fresh-frozen in liquid nitrogen–cooled 2-methylbutane (Fisher Scientific, Pittsburgh, Pa) and stored at −80° C. The small intestine was placed in a metal cassette and embedded in O.C.T. compound (American MasterTech Scientific, Inc, Lodi, Calif), then frozen on dry ice and stored at −80° C. For hematoxylin and eosin staining and immunohistochemistry, 10-μm transverse sections of muscle were cut on a cryostat, mounted on glass slides, and fixed in 70% ethanol for 20 minutes.

Morphologic analysis. To assess the morphology of the tissue samples, the hematoxylin and eosin slides were converted into digital images with a Nikon stereo microscope (Nikon Bioscience Instruments, Melville, NY) connected to a computer. The digital images were analyzed with the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The area was calculated according to the histopathologic differences in the tissue sections, which were classified as viable muscle, inflammation, or necrosis and expressed as a percentage of the total area in each section. Viable muscle contained closely packed, homogenous, and eosinophilc myocytes with peripheral nuclei. Inflammation consisted of cellular infiltration of polymorhonuclear lymphocytes and monocytes (macrophages, lymphocytes, and plasma cells). *Necrosis* was defined as hypereosinophilic muscle fibers lacking nuclei.

Immunohistochemistry. Rat endothelial cells were labeled by incubating sections overnight at 4° C with mouse antirat CD31 monoclonal antibody (1:500; Serotec Inc, Raleigh, NC). CD31 is specific for rat endothelial cells. The slides then were washed in phosphate-buffered saline solution for 5 minutes, and a biotinylated horse antimouse immunoglobulin G (1:250; Vector Laboratories, Burlingame, Calif) was applied for 60 minutes. The slides were again washed, and an avidin-biotin horseradish peroxidase complex (Vector Laboratories) was applied for 60 minutes. They then were rinsed with phosphate-buffered saline solution and colorized with diaminobenzidine (Vector Laboratories). The slides were examined for positive staining of CD31 (reddish brown color) with light microscopy.

A BrdU labeling and monoclonal antibody detection kit (Roche Diagnostics Corp, Indianapolis, Ind) was used to determine cellular proliferation. Briefly, after peroxidase blocking with 0.3% hydrogen peroxide and rinsing in tris-buffered saline solution (TBS), the slides were incubated in 2N HCl for 30 minutes at room temperature. Slides then were rinsed with TBS and blocked with avidin and biotin. The slides were incubated with anti-BrdU mouse monoclonal antibody for 30 minutes at room temperature and then rinsed with TBS. The slides were treated with a biotinylated secondary antibody (horse antimouse [rat absorbed], Vector Laboratories) followed by a streptavidin peroxidase conjugate and developed with diaminobenzidine chromogen. The slides were counterstained with hematoxylin, dehydrated, and mounted. Slides from all groups were run in parallel on the same day. Negative controls consisted of similar sections treated in an identical manner, excluding the primary antibody. Cellular proliferation was quantified with counting of BrdU-stained cells in 10 fields per slide at a magnification of 400×.

Capillary density and capillary-to-muscle fiber ratio. Slides of the left gastrocnemius from five rats of the 24-day ischemia group that showed viable muscle were analyzed in a blinded fashion with a compound microscope (Leica Microsystems, Bannockburn, Ill) and compared with the right gastrocnemius. One rat did not have enough viable muscle to count capillaries. Capillary density was measured with counting capillaries in 10 random fields in a calibrated graticule measuring 0.0625 mm^2 at a magnification of 400×. Capillary-to-muscle fiber ratios were calculated with counting capillaries in five random muscle fiber bundles in blinded fashion at magnification of 200×. The mean results for capillary density (capillaries per mm^2) and capillary-to-muscle fiber ratio are reported.

Statistics. Data are expressed as the mean ± the standard deviation. Statistical comparisons between groups were done with the analysis of variance with Student-Newman-Keuls post hoc test. Statistical comparisons between left and right limbs in the same animals were done with paired Student *t* test. A *P* value of less than .05 was considered statistically significant.

RESULTS

Clinical ischemia index. Immediately after surgery, all rats began limping on the left limb and had an index of 1+. By day 10, the footpad of the left limb in all rats had pressure sores develop and had an index of 2+ (Fig 1). On day 24, the left limb had an index of 1+ and the rat no longer limped. Clinical signs of ischemia appeared to have resolved by day 40 in all rats. The index was zero, the limbs appeared normal, and the rats had a normal gait.

Oxygen measurements. Muscle pO_2, with the rats breathing RA, was significantly lower in the ischemic left limbs than in the normal right limbs at days 3, 10, 24, and 40 after ischemia was induced (Fig 2 , *A*). This difference in pO_2 between the ischemic and normal limb was greater with the rats breathing 100% O_2. Muscle pO_2 was significantly lower in the ischemic left limbs than in the right limbs on days 3, 10, and 40 with rats breathing 100% O_2 (Fig 2 , *B*). On day 24, the muscle pO_2 was lower than that of the control, but the difference did not reach statistical significance. No difference was seen in pO_2 between the nonischemic right legs.

Muscle atrophy. On day 3, the ratio of muscle mass-to-total body weight of the left TA was significantly greater than the right TA as a result of edema and inflammation (0.316 ± 0.029 versus 0.252 ± 0.026; $n = 6$; $P < .05$). Although the mean ratio of muscle mass-to-total body weight of the left gastrocnemius was greater than the right

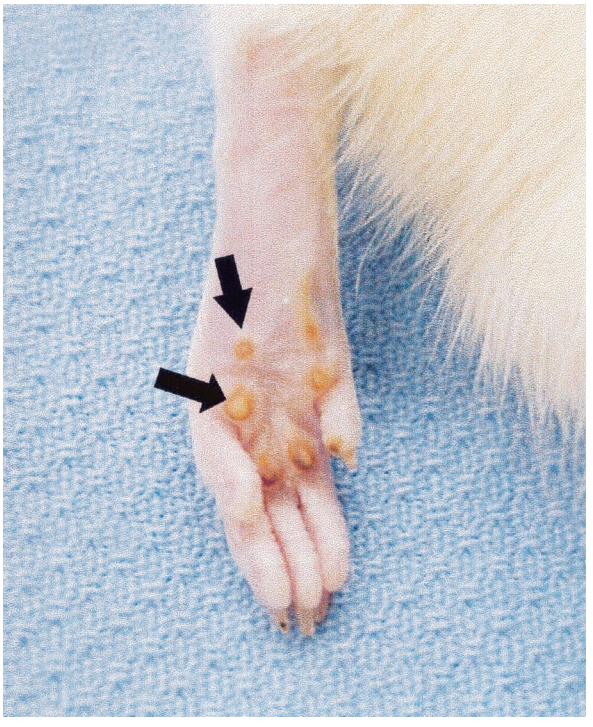

Fig 1. Ischemic paw of rat 10 days after induction of ischemia. Note pale and dusky appearance and pressure sores on foot pads *(arrows)*.

gastrocnemius on day 3, this was not statistically significant (0.744 ± 0.162 versus 0.689 ± 0.026; n = 6; $P > .05$). By days 10, 24, and 40, the muscle masses of the left gastrocnemius and TA were significantly less than the right as a result of muscle atrophy (Fig 3).

Morphology. Tissue sections of the ischemic left gastrocnemius and TA showed the greatest amount of tissue necrosis at 3 days (Fig 4). The sections were characterized by a central area of frank necrosis with interstitial edema surrounded by a band of inflammation that extended peripherally into more normal muscle and was confined to the interstitium (Fig 5, *A*). In these areas were scattered clusters of dying myocytes. Inflammation peaked at 10 days in both muscles, and the necrotic areas were less edematous but comparable in size with the 3-day muscles. Granulation tissue was visible by 24 days and was characterized by monocytes, fibroblasts, and capillaries. Throughout the area of granulation, numerous multinucleated cells were seen with amphiphilic cytoplasm that resembled regenerating muscle fibers (Fig 5, *B*). Inflammation persisted in the TA but decreased in the gastrocnemius by 24 days. Viable muscle gradually increased over time, and by 24 days, the gastrocnemius was nearly fully regenerated and the TA continued to recover from the ischemia. Overall, the necrosis and inflammation were worse in the TA than the gastrocnemius. The right TA and gastrocnemius had normal viable muscle at all time points.

Capillary density and capillary-to-muscle fiber ratio. At 24 days, capillary density was significantly higher in the left limb than in the right limb (834.6 ± 285.1 cap/mm^2 versus 490.9 ± 189.6 cap/mm^2, respectively; $P < .05$). Similarly, the capillary-to-muscle fiber ratio was significantly higher in the left limb than in the right limb (3.1 ± 1.1 versus 1.9 ± 0.6, respectively; $P < .05$).

Cellular proliferation. Cellular proliferation measured with BrdU staining peaked in the left limb at 3 days and was significantly greater than the right limb (146.1 ± 78.55 cells/mm^2 versus 5.6 ± 5.1 cells/mm^2, respectively; $P < .05$). Staining subsequently decreased in the left limb and by 40 days was comparable with the right limb.

DISCUSSION

We have shown chronic severe ischemia in the rat hindlimb up to 40 days with a novel method for measuring tissue ischemia. To our knowledge, this is the first report of oxygen probes being used to measure skeletal muscle pO_2 in a model of hindlimb ischemia. The direct measurement of muscle pO_2 with oxygen probes is a simple method for assessing tissue perfusion[13,14] and correlates with other methods of measuring blood flow.[15]

Using the same animal model in this study, Brevetti et al[15] in our laboratory showed a significant deficit in blood flow measured by fluorescent microspheres up to 24 days after induction of ischemia. Resting blood flow rate was not significantly different in ischemic muscle at rest. However, after exercise, a 10-fold increase and a six-fold increase were seen in blood flow in the normal TA and gastrocnemius, respectively, versus a five-fold and a three-fold increase in the ischemic muscles. The increase in blood flow was blunted by 50% in ischemic muscle compared with normal controls. Exercise was used to place a maximal O_2 and metabolic demand on the muscle and unmask a severe deficit in vascular reserve. This is analogous to the use of 100% O_2 in this study.

Skeletal muscle has a low oxygen requirement but can build up a substantial oxygen debt during exercise.[16] During rest, skeletal muscle blood flow averages only 3 to 4 mL/min/100 g of muscle. During exercise, this can increase 15-fold to 25-fold up to 50 to 80 mL/min/100 g of muscle.[17] Resting muscle pO_2 measurements in the ischemic hindlimb of rats breathing RA on days 10, 24, and 40 showed a reduction in muscle pO_2 of 32%, 26%, and 22%, respectively, compared with the normal limb. This difference in pO_2 was magnified with the rats breathing 100% O_2. With rats breathing 100% O_2 on days 10, 24, and 40, muscle pO_2 measurements showed a 41%, 33%, and 50% reduction, respectively, compared with the normal limbs. The low resting metabolic demand of skeletal muscle may account for the smaller difference in muscle pO_2 recorded while animals were breathing RA. To unmask the true perfusion deficit in the ischemic hindlimb and thereby mimic an exercise state, an oxygen challenge was performed with placing the rats in a sealed chamber with 100% O_2. More than 97% of the body's oxygen content is transported by hemoglobin, and approximately 3% of the oxygen exists

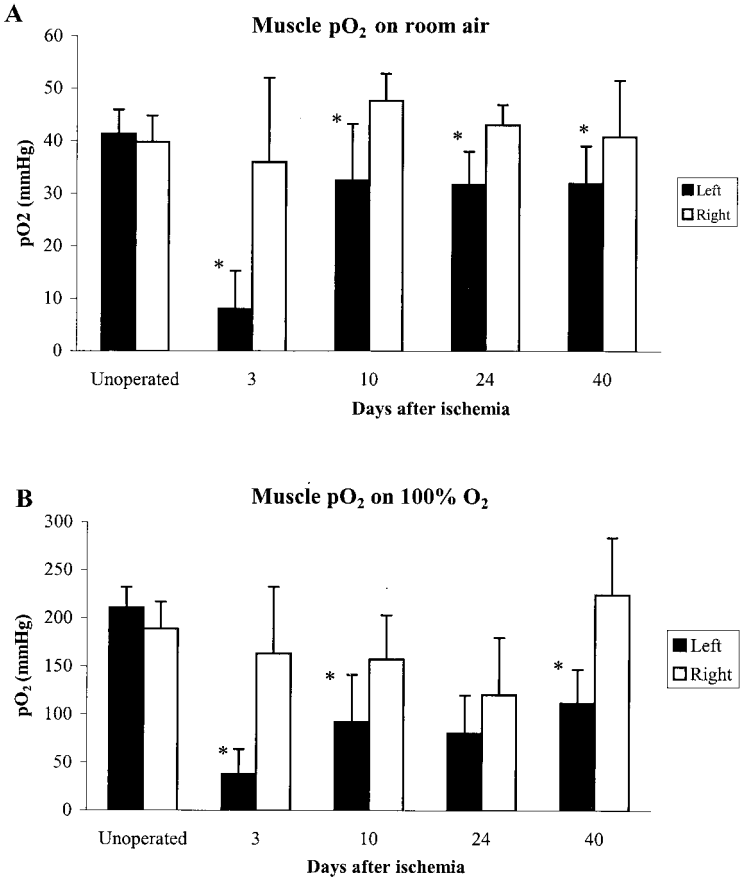

Fig 2. Comparison of muscle pO_2 of left and right limbs on RA **(A)** and on 100% O_2 **(B)**. For RA measurements, n = 6 for unoperated rats, n = 4 at 3 days, and n = 5 at 10, 24, and 40 days. For 100% O_2 measurements, n = 3 for unoperated rats, n = 6 at 3, 10, and 24 days, and n = 3 at 40 days. $*P < .05$ vs. right limb.

in the dissolved state.[16] As the amount of inspired oxygen increases, the dissolved component increases and becomes a significant factor in the O_2 content of blood. An oxygen challenge with 100% O_2 will cause a linear increase in arterial pO_2 in blood and normally perfused tissue. However, if an inadequate tissue blood flow exists because of ischemia, little impact will be seen on arterial pO_2.[12] In our rats, an oxygen challenge unmasked hypoperfusion and ischemia in the operated limb, which was persistent for 40 days. This is in contrast to other models of hindlimb ischemia, which show a more rapid normalization of perfusion.[9,18-21]

Morphologic analysis of the ischemic gastrocnemius and TA documented a sequence of near complete muscle necrosis and regeneration. Although both the gastrocnemius and TA had significant necrosis and inflammation, the TA had greater necrosis and incomplete recovery by day 24. The regeneration was complete in the gastrocnemius by day 24. Fiber type content may have been a factor. The TA consists primarily of fast-twitch glycolytic and fast-twitch oxidative glycolytic fiber types, which have a higher oxygen demand.[17,22] Like the TA, the gastrocnemius consists primarily of fast-twitch glycolytic and fast-twitch oxidative glycolytic but has a larger portion of slow-twitch oxidative fibers types.[22] The predominance of the fast-twitch fiber type and lack of slow-twitch fiber type in the TA creates a linear and parallel capillary network.[23] That the addition of more slow-twitch oxidative fibers as seen in gastrocnemius creates a tortuous capillary network that may improve oxygen delivery and blood supply has been suggested.[23] The combination of a lower oxygen demand and better oxygen delivery may make the gastrocnemius relatively more resistant to ischemia as shown by the greater necrosis and inflammation in the TA.

We observed regeneration of myofibers and an increase in the number of capillaries in the ischemic hindlimb in our model. The induction of skeletal muscle ischemia resulted in an intense inflammatory response, followed by necrosis and subsequent activation of dormant satellite cells. These satellite cells form myotubes that mature into myofibers.[24] It is not widely appreciated that skeletal myocytes are the only adult cells capable of full regeneration. The significance of the regenerative myofibers is shown by the gradual recovery of the muscle mass, especially by 40 days. In a

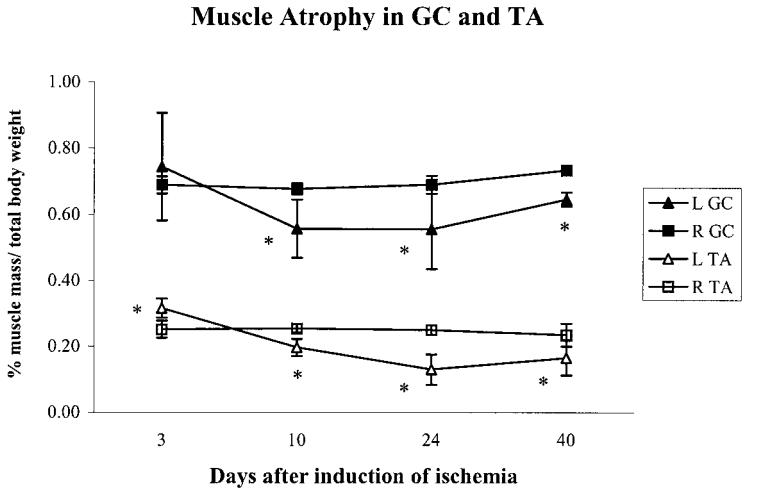

Fig 3. Muscle atrophy represented by ratio of muscle mass to total body weight in gastrocnemius *(GC)* and TA. At 3 days, ratio of muscle mass to total body weight of left TA was significantly greater than right TA as result of edema and inflammation. By 10, 24, and 40 days, left gastrocnemius and TA were significantly less than right as result of muscle atrophy ($*P < .05$).

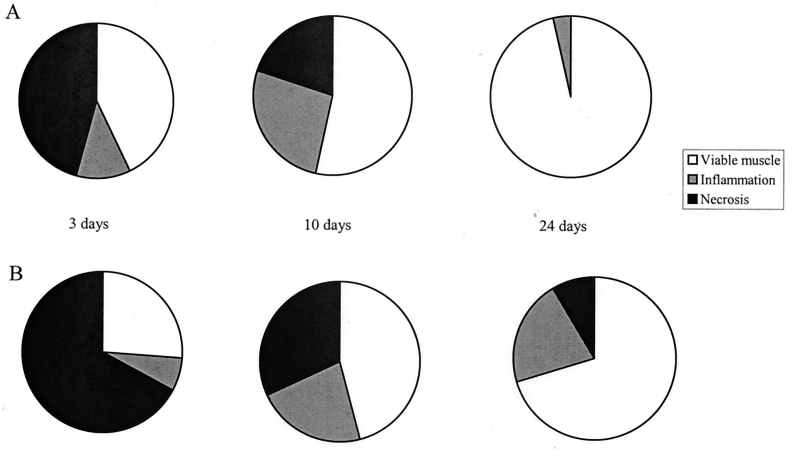

Fig 4. Tissue morphology represented by percent viable tissue versus percent inflammation versus percent necrosis in left gastrocnemius **(A)** and left TA **(B)** at 3, 10, and 24 days after ischemia. Histologic morphology of gastrocnemius and TA showed diminishing necrosis and return of viable muscle over time.

study investigating myofiber regeneration, an increase in the capillary density was noted in the group with the longest duration of ischemia and postischemic period.[25] Our study showed a higher density of capillaries in the ischemic hindlimb than in the normal hindlimb. We also found the capillary-to-muscle ratio to be higher in the ischemic limb, which reduces the likelihood that the higher capillary density of the ischemic muscle was the result of an artifact caused by muscle hypertrophy. This increase in capillary density also is consistent with the increase in cellular proliferation seen in the left limbs. A similar finding of increased capillary density in ischemic muscle was observed by Couffinhal et al[10] in a mouse model of hindlimb ischemia. These investigators suggested that VEGF, which is known to be upregulatd by hypoxia,[26] is responsible for the neovascularization.

Although the density of capillaries in the ischemic gastrocnemius muscle increased, the pO_2 in the ischemic hindlimb did not increase in a parallel manner. This paradox may be explained by failure to develop larger collateral vessels and perfuse the new capillary bed normally. Hershey et al[27] found capillary density to be highest at 5 days after induction of ischemia in the rabbit hindlimb. However, a perfusion deficit persisted until larger collateral vessels developed after 20 days. Capillaries alone are not enough to raise blood flow or tissue pO_2. In addition, other investigators have also noted functional differences of newly formed capillaries. Takeshita et al[28] observed that VEGF-

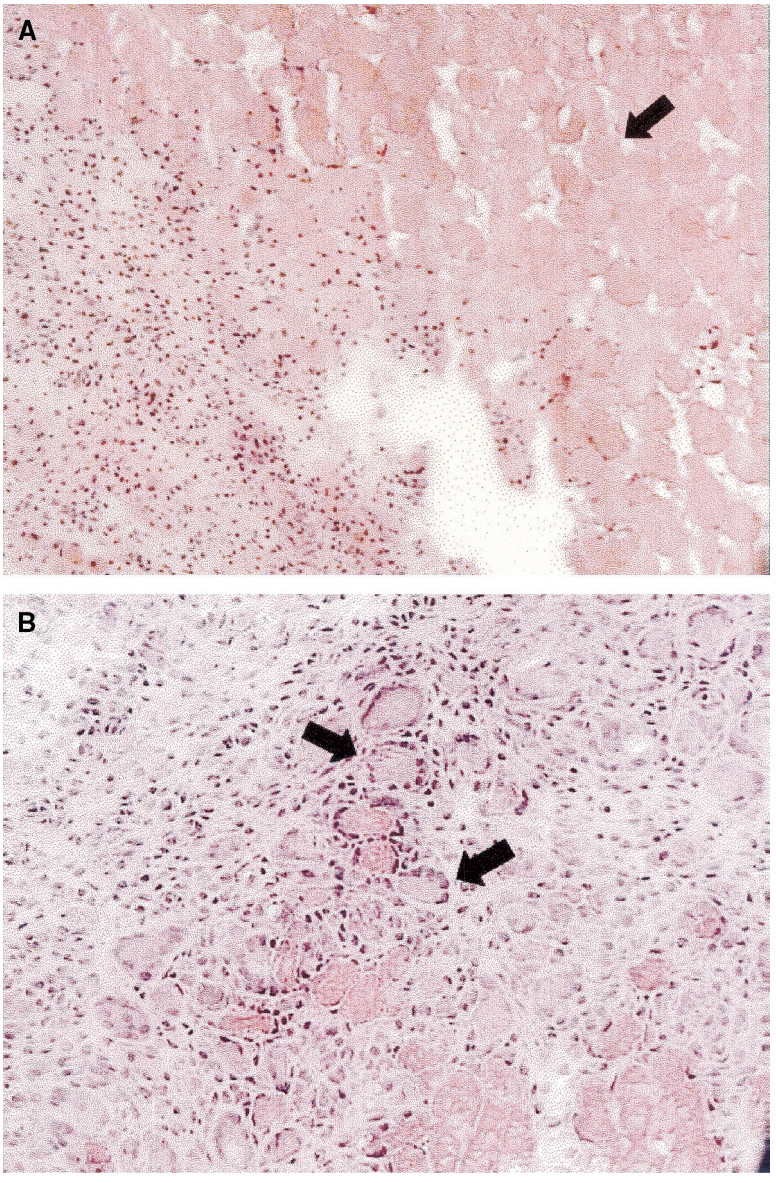

Fig 5. Tissue sections of left gastrocnemius at 10 days **(A)** showing inflammation and dead myocytes (*arrow*; 200×) and left TA at 24 days **(B)** showing granulation tissue with giant cells (*arrows*; 400×).

treated animals had arterioles that dilated more than normal arterioles in response to papaverine hydrochloride, a smooth muscle vasodilator, because of a greater number of smooth muscle cells. Furthermore, the development of newly formed vessels is complex and involves an orchestrated release of growth factors and stabilizing and remodeling proteins.[29,30]

In summary, we have developed a novel rat model of severe chronic hindlimb ischemia characterized by atrophy, necrosis, and partial regeneration of the calf muscles and pressure sores on fat pads of the paws. The significant findings of this study are: 1, ischemia can be quantified with a simple direct measurement of the muscle pO_2, which shows significant hypoxia on RA and on oxygen challenge with 100% O_2; and 2, measurements of skeletal muscle pO_2 correlate with direct measures of blood flow. This severe hindlimb ischemia model may be applicable for future studies of molecular strategies for gene therapy to treat severe limb ischemia in humans.

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